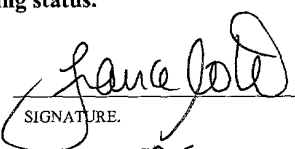


FORM PTO-1390 (REV 10-2000)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER 13045-2US-1 FC	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				U.S. APPLICATION NO. 09/719053	
INTERNATIONAL APPLICATION NO. PCT/CA99/00437		INTERNATIONAL FILING DATE May 13, 1999		PRIORITY DATE CLAIMED June 8, 1998	
TITLE OF INVENTION ACROSOMAL SPERM PROTEIN AND USES THEREOF					
APPLICANT(S) FOR DO/EO/US Robert Sullivan et al.					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
<p>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input type="checkbox"/> This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).</p> <p>4. <input type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))</p> <p style="margin-left: 20px;">a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau).</p> <p style="margin-left: 20px;">b. <input checked="" type="checkbox"/> has been communicated by the International Bureau.</p> <p style="margin-left: 20px;">c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</p> <p>6. <input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).</p> <p>7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))</p> <p style="margin-left: 20px;">a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau).</p> <p style="margin-left: 20px;">b. <input type="checkbox"/> have been communicated by the International Bureau.</p> <p style="margin-left: 20px;">c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</p> <p style="margin-left: 20px;">d. <input type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input type="checkbox"/> An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p> <p><b>Items 11 to 16 below concern document(s) or information included:</b></p> <p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input checked="" type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input checked="" type="checkbox"/> A FIRST preliminary amendment.</p> <p style="margin-left: 20px;"><input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</p> <p>14. <input type="checkbox"/> A substitute specification.</p> <p>15. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>16. <input checked="" type="checkbox"/> Other items or information:</p> <p style="margin-left: 20px;">- Form PCT/IB/308</p> <p style="margin-left: 20px;">- Preliminary Amendment</p> <p style="margin-left: 20px;">- Cover page of WO99/64064</p> <p style="margin-left: 20px;">- Sequence Listing paper and computer-readable form</p> <p style="margin-left: 20px;">- Statement under 1.821 (f)</p>					

U.S. APPLICATION NO. (not yet assigned) <b>09/7119053</b> INTERNATIONAL APPLICATION NO. <b>PCT/CA99/00437</b>		ATTORNEY'S DOCKET NUMBER <b>13045-2US-1</b>	
17. <input checked="" type="checkbox"/> The following fees are submitted: <b>BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :</b> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... <b>\$1000.00</b> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... <b>\$860.00</b> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... <b>\$710.00</b> International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... <b>\$690.00</b> International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) ..... <b>\$100.00</b> <b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>		<b>CALCULATIONS PTO USE ONLY</b>	
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).		<b>\$ 840.00</b>	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	4 - 20 =		X \$18.00
Independent claims	3 - 3 =		X \$80.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$270.00
<b>TOTAL OF ABOVE CALCULATIONS =</b>		<b>\$ 840.00</b>	
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.		<b>\$ 420.00</b>	
<b>SUBTOTAL =</b>		<b>\$ 420.00</b>	
Processing fee of <b>\$130.00</b> for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).		<b>\$</b>	
<b>TOTAL NATIONAL FEE =</b>		<b>\$</b>	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). <b>\$40.00</b> per property		<b>\$ 40.00</b>	
<b>TOTAL FEES ENCLOSED =</b>		<b>\$ 460.00</b>	
		Amount to be refunded:	\$
		charged:	\$
a. <input checked="" type="checkbox"/> A check in the amount of \$ <u>460.00</u> to cover the above fees is enclosed.  b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.  c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>19-5113</u> . A duplicate copy of this sheet is enclosed.			
<b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</b>			
SEND ALL CORRESPONDENCE TO: <b>SWABEY OGILVY RENAULT</b> 1981 McGill College Avenue Suite 1600 Montréal, Québec, Canada H3A 2Y3 No. Tél.: (514) 845-7126 No. Fax.: (514) 288-8389		<div style="text-align: center;">           SIGNATURE  <b>France Côté</b>          NAME  <b>37,037</b>          REGISTRATION NUMBER       </div>	

09/719053

JC01 Rec'd PCT/PTO 07 DEC 2000

PATENT  
13045-2"US"-1 FC/JM

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: **Robert SULLIVAN et al.**  
Application No.: **PCT/CA99/00437**  
Filed: **May 13, 1999**  
For: **ACROSOMAL SPERM PROTEIN AND USES THEREOF**

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents  
BOX PCT  
Washington, DC 20231

Sir:

Prior to calculating the fees pursuant to the entry into National Phase of the above-identified Application please amend the claims as follows.

IN THE SPECIFICATION:

Page 1, line 2, insert the following:

--International application No. PCT/CA99/00437, filed on May 13, 1999, is a continuation-in-part of U.S. priority application No. 09/090,567 filed on June 8, 1998 issued on November 23, 1999 as U.S. Patent No. 5,989,549.--

IN THE CLAIMS:

Please amend claims 1, and 2 now on file as follows:

1. (Amended) A method of [Use of an antigenic fragment of a P34 protein for] immunocontraception of a male or female subject, which comprises administering to said male or female subject an antigenic fragment of a P34 protein to elicit an immunocontraception response by said male or female subject.

2. (Amended) The method [use] according to claim 1, wherein said P34 protein is encoded by a sequence as set forth in SEQ ID NO:3, and wherein said antigenic fragment has an amino acid sequence selected from a group consisting of SEQ ID NO:4 and SEQ ID NO:5.

Claims 1-4 are still in the application.

The above amendment is submitted with respect to Claims 1-4 that were filed with and received by the International Authority on June 24, 2000. No new matter is believed to be introduced herewith.

Applicants believe that the present invention particularly as claimed herein, defines subject matter that is entitled to patent protection in the United States. Accordingly, an early and favorable action on the merits is believed to be appropriate and is courteously solicited.

In the event that there are any questions concerning this amendment, or application in general, the Examiner is respectfully urged to telephone the undersigned so that prosecution of this application may be expedited.

Respectfully submitted,

By:

France Côte, Reg. No. 37,037

Date: December 6, 2000

SWABEY OGILVY RENAULT  
1981 McGill College  
suite 1600  
Montréal, Québec  
Canada H3A 2Y3

(514) 845-7126

ACROSOMAL SPERM PROTEIN AND USES THEREOFBACKGROUND OF THE INVENTION(a) Field of the Invention

5       The invention relates to acrosomal sperm proteins for use in immunocontraception of male and female subjects and as markers for fertility.

(b) Description of Prior Art

10       Fertilization is a highly orchestrated process that culminates in the activation of an oocyte by a spermatozoon. Although testicular spermatozoa are fully differentiated cells, they cannot efficiently encounter the oocyte's investments. In order to acquire this property, spermatozoa must undergo post-testicular  
15       modifications within the epididymis. During this transit, the male gamete is subjected to major surface modifications such as changes in the lipids composition, acquisition of new epididymal proteins as well as post-translational modifications of sperm proteins.  
20       Taken together, these modifications are prerequisites for the spermatozoon to acquire its fertilizing ability. These processes are regulated by the epididymal luminal microenvironment, which is influenced by both epididymal and testicular protein synthesis and secretion.  
25       

30       Using the hamster as a model, a 26 kDa protein, the P26h, which shows immunocontraceptive properties when used to actively immunize male hamsters, has previously been described (Bérubé, B., Sullivan, R., 1994, *Biol. Reprod.*, 51: 1255-1263). This protein is localized on the sperm acrosome and is acquired during the epididymal transit. P26h plays a role in egg-sperm interactions as shown by the ability of P26h antibodies to inhibit sperm-zona pellucida binding in vivo and in  
35       vitro (Bérubé and Sullivan, *supra*).

It would be highly desirable to be provided with an acrosomal sperm protein for use in immunocontraception of male and female subjects.

It would also be highly desirable to be provided  
5 with an acrosomal sperm protein for use as a marker for fertility.

#### SUMMARY OF THE INVENTION

One aim of the present invention is to provide a  
10 use of an acrosomal sperm protein in immunocontraception of male and female subjects.

In accordance with the present invention there is provided a method of immunocontraception of a male or female subject, which comprises administering to the  
15 male or female subject an antigenic fragment of a P34 protein showing a high specificity for the P34 protein to elicit an immunocontraception response by the male or female subject, the antigenic fragment of P34.

The P34 protein used may have a sequence identified as SEQ ID NO:3 and a preferred antigenic fragment  
20 thereof includes, without limitation, MELFLAGRRVL (SEQ ID NO:4) and CHKAKTMLNRI (SEQ ID NO:5).

In accordance with the present invention, there is also provided an immunocontraceptive vaccine for a  
25 male or female subject, which comprises an antigenic fragment of a P34 protein in association with a suitable pharmaceutically acceptable carrier, wherein the vaccine elicits an immunocontraception response by the male or female subject after its administration.

30 In accordance with the present invention there is further provided a probe as a marker for male or female fertility, which comprises a cDNA sequence capable of hybridizing under stringent conditions with the P34 human acrosomal sperm protein.

In accordance with the present invention there is further provided a method for the diagnosis of male or female infertility, which comprises the steps of determining the amount of human P34 protein in a sperm or ovule sample, and comparing the determined amount  
5 with a fertile control sample.

The amount of human P34 may be determined using an antibody raised against the human P34 protein.

In accordance with the present invention there  
10 is further provided a kit for the diagnosis of male or female infertility, which comprises an anti-P34 antibody enzyme-labeled, an enzyme substrate and a fertile control sample.

A calibration curve for the amount of human P34  
15 may be obtained using the fertile control sample.

For the purpose of the present invention, the expression "antigenic fragment" is intended to mean any fragment of the protein that is capable of eliciting an immune response pursuant to its administration to a  
20 subject.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the comparison of partial amino acid sequences with the corresponding amino acid  
25 sequence of P26h with AP27 (adipsin);

Fig. 2 illustrates a Northern blot analysis of hamster total RNA from 1) testis 2) whole epididymis 3) caput epididymis 4) corpus epididymis 5) cauda epididymis 6) fat 7) lung 8) heart 9) liver 10) kidney 11)  
30 muscle and 12) brain, probed with a nP26h 710 bp cDNA probe (upper panel) or with a positive Cyclophilin probe (lower panel);

Figs. 3A-3C illustrate the nucleotide sequence of the P26h cDNA (SEQ ID NO:1) with its encoded protein  
35 (SEQ ID NO:2);

Figs. 4A-4B illustrates the alignment of the deduced amino acid sequence of P26h with the AP27 (adipsin) and the carbonyl reductase;

Fig. 5 illustrates an *in situ* hybridization probed with the P26h RNAs probes;

Fig. 6 illustrates the immunoprecipitation of P26h cDNA translational products;

Fig. 7 illustrates a Northern blot analysis of human total RNA from 1) testis, 2) caput epididymidis, 4) corpus epididymidis and 5) cauda epididymidis, probed with a P34H cDNA probe; and

Figs. 8A-8B illustrates the sequence homology of the human P34 (lower lane) counterpart of P26h (upper lane).

#### DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, the determination of the origin and of the sequencing of the encoding cDNA of the P26h sperm protein showing male contraceptive properties was undertaken. N-terminal sequencing of purified P26h and of peptides generated by partial proteolysis allowed a partial identification of the protein. The Northern blot analysis revealed that a major transcript encoding P26h was localized in the testicular mRNA and that no signal was detectable in other somatic tissues of the hamster. A hamster testis cDNA library was screened and a P26h encoding cDNA was cloned and sequenced. The P26h cDNA sequence revealed a 85% identity with the cDNA corresponding to mouse adipsin and to a carbonyl reductase. The deduced P26h amino acid sequence possesses specific domains of the Short Chain Dehydrogenase/Reductase (SDR) family proteins. Antibodies generated against synthetic peptides deduced from the cDNA sequence recognized the P26h on Western blots of detergent-



extracted hamster sperm proteins. On the other hand, *in vitro* translational products synthesized from the P26h cDNA were immunoprecipitated by a polyclonal antiserum produced against the purified hamster sperm P26h. *In situ* hybridization performed on tissues from the hamster reproductive tract revealed that the P26h was principally transcribed in the seminiferous tubules and at a lower level in the corpus epididymidis. P26h shows unique features of the SDR family that can be used to induce contraception in males.

Sexually mature Golden hamsters (*Mesocricetus auratus*, Charles River Inc., St. Constant, Qc, Canada) were sacrificed under CO<sub>2</sub> atmosphere and the epididymidis excised, defatted and dissected into *caput*, *corpus* and *cauda* segments. Tissues were frozen in liquid nitrogen and stored at -80°C until used. Testicular and somatic tissues were proceeded the same way. For *in situ* hybridization, fresh tissues were rinsed in PBS-DEPC (Phosphate buffered saline-Diethyl pyrocarbonate) and fixed at 4°C for 2h in 4% (w/v) paraformaldehyde freshly prepared in PBS. Tissues were cryoprotected by sequential incubations in 10% glycerol for 1h at 4°C under agitation and then overnight in 50% OCT. Tissues were embedded in OCT and frozen in liquid nitrogen. Cryosections of -7µm were collected on poly-L-Lysine coated slides, air-dried at -20°C and were stored at -80°C until used.

#### **N-chlorosuccinimide proteolysis**

Proteins from *cauda* epididymal spermatozoa or from the epididymal fat pad were extracted with 0.5% Nonidet™ P40 (Sigma) and submitted to a preparative SDS-PAGE. After Coomassie blue staining, the bands corresponding to a MW of 26 kDA were excised, washed twice with H<sub>2</sub>O and rinsed with a washing solution (50% (wt/vol) urea, 50% (vol/vol) ethanol). The polyacryla-

5      mid bands were incubated 30 min. in 20 mg/ml N-Chloro-succinimide in washing solution, washed in water and incubated 3 times for 1h each in an equilibrium solution (0.0625M Tris-HCl pH 6.8, 20% (vol/vol) glycerol, 30% (vol/vol) B-mercaptoethanol, 6% (wt/vol) SDS). The bands were loaded on a discontinuous polyacrylamide gel and submitted to electrophoresis. Patterns of protein fragments were visualized by silver nitrate staining or Western blotted using a P26h antiserum (Bérubé and Sullivan, *supra*). Western blotted P26h fragments were also used for N-terminal sequencing.

#### Partial amino acid sequence analysis

15      P26h was purified and absorbed on a piece of nitrocellulose sheet. 100  $\mu$ l of 50 mg/ml CNBr (Cyanogen Bromide) in 70% formic acid was added to 1 mg of the dry protein and incubated under nitrogen in the dark for 24h. Digested peptides were loaded onto a VYDAK™ reversed-phase C18 column (250 x 1 mm) which was equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA) in water and eluted with a 2-100% gradient of 0.08% (v/v) TFA in 80% acetonitrile. Fractions of 0.5 ml or smaller were collected at a flow rate of 50  $\mu$ l/min. Protein sequencing was performed on aliquots from one peak by automated Edman™ degradation with a pulsed-liquid phase sequencer.

#### RNA extraction

30      Tissues were homogenized with a Polytron™ in 1.5 ml of a fresh homogenization buffer solution (4M guanidium thiocyanate, 25mM sodium citrate pH 7, 0.5% sarcosyl, 0.1M 2-mercaptoethanol). 1 ml of Cesium Chloride-homogenization buffer (2g of CsCl/2.5 ml) was added to the tissue lysates. This was layered on cushion solution (5.7M CsCl, 0.1M EDTA, pH 7.5) and centrifuged at 60 000g overnight. The RNA pellets were resuspended in TES solution (10mM tris-HCl, 5mM EDTA, 1%

35

SDS, pH 7.4) and extracted with phenol/chloroform and chloroform/alcohol isoamyl 24:1. The RNA was precipitated with 0.1 vol. of sodium acetate (3M, pH 5.2) and 2.5 vol. of ethanol 95%. The RNA pellets were resuspended in DEPC water. The RNA quality was evaluated by electrophoresis on a 1% agarose gel. All solutions were made with DEPC water.

#### Northern blot analysis

The total RNA (20 µg) prepared from hamster and human tissues was electrophorized on 1% agarose-formaldehyde gels and transferred to a nylon membrane (Quiagen, Santa Clarita, CA) using 20x SSC (3M NaCl, 0.3M Na-Citrate). Air dried Northern blots were UV cross-linked and prehybridized at 42°C for 4h in 50% (vol/vol) formamide, 0.75 M NaCl, 0.05 M NaH<sub>2</sub>PO<sub>4</sub>, 0.005M EDTA, 2 X Denhardt's reagent [0.2% (wt/vol) Ficoll 400, 0.2% (wt/vol) polyvinylpyrrolidone, 0.2% (wt/vol) BSA], 0.2 mg/ml herring sperm DNA (Sigma Chemicals, Mississauga, ON) and 0.1% SDS. The membrane was hybridized overnight at 42°C in the same solution, to which [ $\alpha$ -<sup>32</sup>P] dCTP-labeled DNA probes were added. The membranes were then washed twice in 0.1 x SSC-0.1% SDS followed by a third wash of 30 min. at 65°C in 0.1 x SSC-0.1% SDS, and exposed on Kodak™ X-O-Mat film with intensifying screens for 6-18 h at -80°C. A RNA ladder (1.6-7.4 kb; Boehringer Mannheim, Laval, QC) was electrophorized in parallel and Cyclophilin probe was used as a constitutive internal control.

#### RT-PCR production of a P26h cDNA probe

The first amino acids sequence obtained (MKLNFSXLRLVTGAGKGIG) showed a high homology with the peptide sequence of adipsin, a marker of adipocytes differentiation. From the nucleic acid sequence of adipsin, two primers were selected according to OLIGO 4.01™ primer analysis software (National Biosciences,

Plymouth, MN), chemically synthesized (sense downstream 5'-GTG ACA GGG GCA GGG AAA GGG-3' (SEQ ID NO:6) and antisense upstream 5'-GCA ACT GAG CAG ACT AGG AGG-3' (SEQ ID NO:7)) and used for RT-PCR on the total RNA  
5 from hamster testis.

Briefly, 5 µg of the total testis RNA was incubated with 0.5 µg oligo deoxythymidine primer at 70°C for 10 min. in a final volume of 12 µl and kept on ice. Samples were incubated for 60 min. at 42°C in a reaction  
10 mixture containing 4 µL of 5X buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl<sub>2</sub>), 10 mM dithiothreitol (DDT), 1.25 mM deoxynucleotide triphosphates (dNTP) and 200 IU Super Script reverse transcriptase in a final volume of 20 µl. Expression of the P26h gene was determined by  
15 amplification of the cDNA. Each reaction contained 5 µl of RT template (or water as negative control), 1.5 mM MgCl<sub>2</sub>, 1 x buffer, 0.2 mM dNTPs, 10 µM of each primer and 1.5 U Taq polymerase (Pharmacia Biotech, Baie D'Urfé, QC) in a final volume of 50 µl. The PCR cycling  
20 conditions chosen were 1 min. at 95°C, 1 min. at 60°C, 1 min. at 72°C for 30 cycles, followed by a 5 min. extension at 72°C. The reaction products were analyzed using electrophoresis on a 1% agarose gel and the bands were visualized by ethidium bromide staining.

The PCR band (~710 bp) was purified (Quiaquick; Quiaagen), T-Cloned in pCR 3.5 (Invitrogen, San Diego, CA), and digested with EcoR1. The insert (710 bp) was separated from the vector and other fragments by electrophoresis on a 1% agarose gel, isolated from gel  
30 matrix with a Na45 membrane (Schleicher & Schuell, Inc.), and random-prime labeled according to the supplier's instructions using the T7 Quick-Prime™ kit (Pharmacia Biotech, Baie D'Urfé, QC) with [α-<sup>32</sup>P] dCTP. Cyclophilin cDNA was also random-prime labeled using  
35 the same procedure.

- 9 - 14 24.08.00

**Cloning and sequencing of P26h cDNA**

Poly(A)<sup>+</sup>RNA from hamster and human testicular tissues was purified from total RNA solution using a poly(A)<sup>+</sup>RNA purification kit (Pharmacia Biotech, Baie D'Urfé, QC) according to the supplier's instructions. The cDNA library was prepared according to the supplier's instructions. Briefly, testicular poly(A)<sup>+</sup>RNA was reverse-transcribed and ligated into the lambda Uni-Zap<sup>TM</sup> XR vector (Stratagene, La Jolla, CA). The lambda library was packaged and amplified using *Escherichia coli* XL1-Blue cells and screened with the random-prime labeled 710 bp P26h cDNA. The positive clones were isolated by plaque purification and the longest one (1081 bp) was subcloned into pBluescript KS<sup>+</sup><sup>TM</sup>. All nucleotide sequences were determined by the dideoxynucleotide termination method (Sanger) using T7 Sequenase v 2.0 kit. The labeled reaction products were analyzed on a DNA sequencer gel. Sequence translation was performed using Gene Jockey software (Biosoft, Cambridge, UK).

**In situ hybridization**

Tissue cryosections were fixed with freshly prepared 4% paraformaldehyde in PBS for 5 min. at RT (room temperature), incubated for 10 min. in 95% ethanol/5% acetic acid at -20°C and rehydrated by successive baths of decreasing concentrations of ethanol diluted with DEPC-H<sub>2</sub>O. Target RNA was unmasked by enzymatic digestion with 10 µg/ml proteinase K (Boehringer Mannheim) in PBS for 10 min. at 37°C, followed by a 5 min. incubation in 0.2% glycine. Sections were postfixed for 5 min. with 4% paraformaldehyde in PBS, acetylated with 0.25% acetic anhydride, 0.1 M triethanolamine, pH 8.0, for 10 min. and finally washed with PBS.

Tissues were prehybridized for 1h with a preheated 250 µg/ml salmon sperm DNA in a hybridization

5 solution (0.3M NaCl, 0.01M Tris-HCl pH 7.5, 1mM EDTA, 1x Denhardt's solution, 5% dextran sulfate, 0.02% SDS and 50% formamide). Sections were then incubated overnight at 42°C, under coverslips, with 25µl of heat-denatured antisense or sense cRNA probed with DIG (Digoxigenin: Boehringer Mannheim) according to the supplier's instructions. Coverslips were removed, the sections were washed twice in 2x SSC at RT, followed by two 10 min. washes at 42°C in 2x SSC, 1x SSC and 0.2x 10 SSC, respectively.

Hybridization reactions were detected by immunostaining with alkaline phosphatase-conjugated anti-DIG antibodies. Nonspecific staining was blocked by incubation for 1h with 5% (v/v) heat-inactivated sheep 15 serum in 0.2M Tris-HCl, 0.2M NaCl, and 3% Triton™ X-100. Sections were then incubated for 2h at RT with the alkaline phosphatase-conjugated anti-DIG antibodies diluted 1:1000 in blocking solution, washed with tris-HCl/NaCl buffer, and incubated with 0.1M tris-HCl, pH 20 9.5, 0.1M NaCl, and 0.01M MgCl<sub>2</sub>. The hybridization signal was visualized after a 10-15 min. incubation with the substrates nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (GIBCO-BRL, Gaithersburg, MD). Levamisole (2 mM; Sigma) 25 was added to the reaction mixture to inhibit endogenous alkaline phosphatase. Slides were immersed in 1mM EDTA, 0.01 Tris-HCl, pH 7.5, washed 5 min. in H<sub>2</sub>O, counterstained with neutral red, dehydrated through baths of ethanol, cleared in xylene and mounted with Permount 30 (Fisher scientific, Nepean, Ontario, Canada).

#### **Eukaryotic in vitro translation**

*In vitro* translation was performed from circular plasmid DNA including the P26h cDNA. The TNT coupled reticulocyte lysate system was used according to the 35 supplier's instructions (Promega, Madison, WI, USA).

Briefly, 0.5  $\mu$ g of circular plasmid DNA was added directly to TNT rabbit reticulocyte lysate. T3 RNA polymerase (Promega, Madison, WI, USA) and S<sup>35</sup>-methionine (10 mCi/ml) were added to the translation mixture.

5 The reaction was performed for 2h at 30°C. The *de novo* synthesized proteins were analyzed by SDS-PAGE according to Laemmli. The gel was soaked in an enhancer solution (Amersham), dried and exposed on X-Omat<sup>TM</sup> AR film (Kodak) for 6h at RT. In some experiments, the translational products were submitted to NCS proteolysis before electrophoretic analysis.

In some experiments, the translational products were immunoprecipitated using an anti-P26h antiserum. 5  $\mu$ l of the translation reaction mixtures were incubated 15 1h at RT with the P26h antiserum (Bérubé and Sullivan, *supra*) or the control serum, both diluted in Tris-saline (50mM Tris-HCl, 150mM NaCl, pH 7.5). 50  $\mu$ l of packed protein-A sepharose (Pharmacia) was added for 1h at RT. The immunoprecipitate was washed several times 20 in tris-saline (50mM Tris-HCl, 500mM NaCl, pH 7.5) solution. The immune complexes were dissociated in SDS-PAGE sample buffer (50 mM Tris-HCl pH 6.3, 2% SDS (w/v) and 5% (v/v) B-mercaptoethanol) and submitted to SDS-PAGE according to Laemmli. The gel was enhanced, dried 25 and exposed on X-Omat<sup>TM</sup> AR film (Kodak) for 12h at RT.

The hamster's epididymis, like that of many mammalian species, is surrounded by a fat pad. In the mouse, an adipsin mRNA is abundant in the epididymal fat cushion. There was a possibility that P26h N-terminal sequences obtained may result from a contamination 30 of sperm protein preparation by the epididymal fat pad originating adipsin. This was conceivable if we consider that the adipsin MW deduced from the mRNA sequence is of 27 kDa. A band corresponding to 26kDa of 35 an electrophoretic pattern of protein extracted from a

large amount of epididymal fat cushion was excised and submitted to N-chlorosuccisinimide proteolysis (NCS). This digestion did not generate fragments on the SDS-PAGE electrophoretic pattern whereas the P26h NCS digest generated a 22.4 kDa fragment. Moreover, only the P26h and its NCS digested fragment were detected by a P26h antiserum used to probe a corresponding immunoblot. The NCS digested fragment of the P26h was sequenced and the inner sequence revealed also a high homology level with the Adipsin. The intact 26 kDa from the fat pad protein extract was submitted to the same procedure and no N-terminal sequence was obtained.

RT-PCRs were performed with oligonucleotides derived from the cDNA sequence of the adipsin. A 710 bp fragment was amplified from the hamster testis, cloned and sequenced. Using this fragment as a probe, a Northern blot analysis was performed to determine in which tissues P26h transcription occurs. Total RNA of several tissues were extracted and submitted to blot-hybridization analysis. The Northern blots showed that the total P26h messenger RNA has 1081 bp and that it was transcribed exclusively in the testis. To confirm the presence of intact RNA in all samples, the same blot was probed with random cyclophilin DNA, and an intense signal was obtained in all samples (Fig. 2). By opposition, Northern blot analysis of mRNA prepared from human tissues revealed an abundant transcript in the epididymal tissues (Fig. 7).

A cDNA library was constructed in Lambda gt11 from the hamster testicular mRNA and from the human epididymal tissues.  $10^5$  clones of the primary library ( $5 \times 10^5$  clones) were directly screened with the 710 bp cDNA probes. The first screening allowed the detection of 32 positive clones from which 11 were used for a second and third screening. The size of the inserts was



determined by PCR and the longest insert (clone 2), was introduced in pBluescript SK(+/-) phagemid and sequenced. The P26h cDNA of 1081 bp has a 732 bp open reading frame (orf) starting with a ATG codon at position 124 and a TAG stop codon at position 856, followed by a poly-adenylation signal, and a poly A tail (Figs. 3A-3C). The sequence is numbered from the 5' end of the cDNA clone. The translation of the proposed orf is shown below the nucleotide sequence and encodes a peptide of 244 amino acids terminating by an amber codon.

### Results

When purified P26h was submitted to Edman degradation, 27 of the 29 amino acids generated were identified. A 17 kDa fragment obtained by NCS proteolysis of P26h allowed the identification of 15 of the 26 amino acids analyzed, whereas the fragment obtained following CNBr treatment allowed the identification of 8 of 9 additional amino acids. For a total of 40 amino acids identified by Edman degradation of P26h peptides, 37 showed homology with a mouse adipsin sequence (Fig. 1). This protein has been shown to be a differentiation growth factor of mouse adipoblasts.

The deduced amino acid sequence predicted a 26 kDa MW protein which is in agreement with the mw of the P26h as determined by SDS-PAGE. The N-terminal sequence of P26h and of its generated peptides determined by Edman degradation (Fig. 1) are also in agreement with the amino acid sequence deduced from the cDNA (Figs. 4A-4B). The P26h amino acid sequence was compared with adipsin and a carbonyl reductase, which showed a homology of 85% and 86% respectively. The deduced amino acid sequence of the human homologue (Figs. 8A-8B, lower sequence) predicts a 209 amino acid peptide sharing the SDR characteristics with the hamster P26h (Figs. 8A-8B, upper sequence).

Expression of P26h mRNA is detected in the testis using non-radioactive *in situ* hybridization. Digoxigenin-labeled anti-sense probe revealed the expression of the P26h mRNA in the adult hamster testicular seminiferous tubules. By opposition to the Northern Blot analysis, *in situ* hybridization revealed a weaker signal along the epididymis, principally in the *corpus* portion. Digoxigenin-labeled sense probe was used as a control for nonspecific hybridization. The *in situ* hybridization confirms that a P26h transcript is predominant in the testis and, at a lower level, in the epididymis. *In situ* hybridization has been performed with digoxigenin-labeled RNA probes system using an anti-digoxigenin antibody that allows amplification of the signal and provides a more sensitive mRNA detection than the traditional Northern blot analysis. A faint labeling is detectable all along the epididymis, a much stronger signal being associated with the *corpus* (Fig. 5). In many species, the *corpus* region is known to be a more active epididymal segment for protein synthesis and secretion. According to the Northern blot analysis (Fig. 2), P26h which is found at high concentration in the proximal region of the hamster epididymis probably originates from the testicular fluid as a secretion product of the seminiferous tubules, as suggested by the *in situ* localization of the transcript (Fig. 6). This protein may also be secreted by the *corpus* epididymidis. A dual testicular and epididymal origin has been described for other proteins interacting with the spermatozoa during epididymal maturation. Whether the testicular and the epididymal P26h are identical or exist in different isoforms, as described for clusterin, remains to be determined.

Using the TNT<sup>TM</sup> coupled reticulocyte lysate system, *in vitro* translation was performed with circular

plasmid including P26h cDNA. A 26 kDa signal with total translation products was detected on SDS-PAGE. (Fig. 6A3). Total translation products were then submitted to immunoprecipitation with anti-P26h antibody, which permitted the detection of a unique signal of 26 kDa on SDS-PAGE. (Fig. 6A2). Total translation products were further submitted to NCS proteolysis. The NCS proteolysis generated a 17 kDa fragment on SDS-PAGE in agreement with the deduced amino acids sequence and the previous NCS proteolysis of purified P26h.

### Discussion

One of the best documented physiological functions acquired by the spermatozoa during epididymal maturation is their ability to efficiently interact with the egg's zona pellucida. The inventors have been interested by these sperm surface modifications, mainly the addition of new surface proteins or the post-translational modifications of preexisting sperm components that are necessary to produce a functional male gamete. P26h is abundant in the luminal fluid of the proximal region of the hamster epididymis, its concentration decreasing along the transit. Contemporarily, P26h accumulates on the spermatozoa during the epididymal maturation. P26h is exclusively located on the sperm surface covering the acrosomal cap of the mature spermatozoa, the subcellular domain involved in zona pellucida binding.

A single spot in two dimensional gel electrophoresis was obtained, this single protein being recognized by the anti-P26h on corresponding Western blot. These two preparations of purified P26h, as well as proteolytic fragments, have been N-terminal sequenced by Edman degradation. All the amino acid sequences obtained showed high homology with mouse adipsin (Fig. 1). Adipsin mRNA has been shown to be present in high

quantities in the mouse epididymal fat pad. Proteins from huge amounts of epididymal fat pad were extracted and proceeded in parallel with cauda epididymal spermatozoa. Protein bands of 26-27 kDa were excised from preparative SDS-PAGE of proteins extracted from fat pad adipocytes and from cauda epididymal spermatozoa. Intact 26-27 kDa bands and proteolytic fragments generated by NCS (N-Chlorosuccinimide) digest were Western blotted and probed with the anti-P26h serum. The 26-27 kDa fat pad protein was undetectable with the anti-P26h antiserum (Fig. 2). Furthermore, the 26-27 kDa fat pad band and the P26h sperm protein were proceeded in parallel for N-terminal sequencing by Edman degradation. No signal was detectable when the fat pad protein was proceeded. From these results we can conclude that the N-terminal sequences obtained did not result from a contamination of sperm preparation by the epididymal fat pad.

Northern blot analysis reveals a major P26h transcript in testicular tissues of the sexually mature hamster (Figs. 3A-3C). This mRNA is undetectable in the other tissues analyzed, including the fat pad and the epididymis (Figs. 3A-3C). This was unexpected since it was previously reported that an *in vitro* translational product encoded by mRNA of the proximal region of the epididymis can be immunoprecipitated by anti-P26h antibodies.

The P26h being transcribed principally in testicular tissues (Fig. 2), a testicular cDNA was screened to clone the P26h cDNA. The longest transcript obtained from the library was sequenced and revealed a cDNA of 1081 bp coding for a 244 amino acids protein. The P26h cDNA shows high sequence homology with adipsin, as expected from N-terminal amino acid sequences and with a carbonyl reductase known to be expressed in

0020267 65061600

pig lung (Figs. 4A-4B). The deduced amino acid sequence also shows a high homology of 87% with the adipsin and 80% with the carbonyl reductase. Considering that P26h is a sperm protein involved in gamete interactions, the biological function of these two proteins was puzzling. Adipsin has been described as a differentiation factor of adipoblast in adipocytes and its expression has been shown to be inhibited by activators of protein kinase C. The carbonyl reductase is a homotetramer that catalyzes the oxidation of secondary alcohols and aldehydes. This enzyme has been shown to be expressed specifically in the lung and mainly distributed in the mitochondria. Notwithstanding the high level of homology with adipsin and carbonyl reductase, P26h shows a complete different tissue distribution. The P26h protein and its encoding mRNA are not expressed in the lung nor in the adipocyte (Fig. 2; Bérubé and Sullivan, *supra*). Adipsin and carbonyl reductase are known to be members of the short-chain dehydrogenase/reductase (SDR) superfamily and P26h shows some of their properties.

The Short-Chain Dehydrogenase/Reductase superfamily (SDR) is formed by a variety of different proteins that exhibit residue identities of only 15-30%. This low level of sequence identity between the members indicates an early divergence. This is reflected by the wide range of functions fulfilled by the members of this superfamily. There are three classes of enzymes covering a wide range of EC numbers: 1, 4.2, 5.1, and 5.3, as well as members with unknown functions. Two consensus sequences are conserved in this family, the NAD(H) or NADP(H) binding domain, a N-terminal segment GlyXXXGLYXXGly, and the catalytic domain having the sequence TyrXXXLys. The P26h deduced amino acid sequence possesses these consensus domains as well as

the Gly 129, Ser 136 and Pro 179, which are conserved in more than 90% of the SDR family members (Figs. 4A-B).

Polyclonal antibodies have been produced against P26h and used to document the function of this sperm  
5 protein during the fertilization processes in the hamster. When added to an *in vitro* fertilization medium, the antibodies anti-P26h inhibit in a dose-dependent manner the sperm-zona pellucida interaction. Furthermore, active immunization of male hamsters against the  
10 purified P26h results in an immune response associated with reversible infertility. Using the anti-P26h antiserum, a human counterpart of P26h has also been identified and was showed to be absent from sperm of men presenting idiopathic infertility. In humans, this  
15 protein is also acquired by the spermatozoa during the epididymal transit. Taken together, these results clearly demonstrate the involvement of this sperm protein in the processes leading to fertilization. The P26h preparation that shows immunocontraceptive proper-  
20 ties is the same than the one used to determine N-terminal sequence by Edman degradation (Fig. 1). Furthermore, the polyclonal antiserum that allowed us to document the function of P26h in the processes of fertilization also reacts with the translational product  
25 encoded by the sequenced cDNA (Fig. 6). This clearly demonstrates that this SDR member is involved in mammalian sperm-egg interaction.

The mammalian spermatozoon is a highly polarized cell characterized by well-defined membrane domains.  
30 Many sperm surface proteins have been proposed to play a role during the cascade of events occurring when the male gamete reaches the oocyte. Different sperm proteins have been proposed as candidates involved in zona pellucida binding. Some of them show enzymatic activity  
35 such as proacrosin, a trypsin like protease, a mannosid-

dase, a galactosyltransferase and P95, a hexokinase. The catalytic activity of these enzymes may not necessarily be involved in *zona pellucida* interaction; it is rather the substrate affinity that mediates this interaction. The biological function played by these proteins in gamete interactions is thus quite different from their enzymatic activity defined by their catalytic activity in cell metabolism. This discrepancy is reflected by their subcellular localization on the spermatozoon. To mediate *zona pellucida* recognition these enzymes must be localized at the sperm surface where they are classically known to be intracellular. This is well illustrated by the extracellular oriented sperm membrane mannosidase and galactosyltransferase, as well as by hexokinase, which is at the surface of the mouse spermatozoa where it is known to be associated with the mitochondrial membrane. Like these potential *zona pellucida* ligands, P26h is localized at the hamster sperm surface, to the membrane domain covering the acrosome.

P26h belongs to the SDR superfamily, which is characterized by highly different members with a low level of identity. This reflects distant duplications and early divergence. As a consequence, the SDR family represents a great diversity in enzymatic activities and functions. An interesting example of an alternative function for an enzyme is glyceraldehyde -3-phosphate dehydrogenase. This protein, which is classically known as a glycolytic enzyme, has been shown to act as a t-RNA binding protein with a function in cytoplasmic trafficking. SDR divergence is favorable for the arising of new functions, and involvement in gamete interactions may be one of these.

Considering that P26h has been previously shown to be involved in gamete interaction and to possess

immunocontraceptive properties, cloning of a homologous cDNA in human allowed the identification of a human sperm protein with immunocontraceptive properties (Figs. 8A-8B).

5       The blood-testis barrier is not present in the epididymis, allowing the neutralization of spermatozoa following immunization against an antigen involved in post-testicular maturation of the male gamete. The fact that the human sperm protein is specifically expressed  
10   in the epididymis (Fig. 7) strongly supports its potential as an immunocontraceptive target.

      The present invention will be more readily understood by referring to the following example, which is given to illustrate the invention rather than to  
15   limit its scope.

#### EXAMPLE I

##### **Immunocontraception vaccine**

      The human counterpart of the hamster cDNA P26h  
20   encodes for an epididymal-specific protein that is important in sperm function. It is possible to target this protein by specific antibodies using an immunocontraceptive approach. Men may be immunized with a peptide corresponding to the epididymal protein. This pep-  
25   tide may be chosen with regards to its antigenic properties. An immune response against that specific peptide may occur and with no side effects since the selected peptide shows high specificity for a sperm-epididymal protein. The antibodies may reach the sper-  
30   matozoa within the excurrent duct (epididymis) since the blood-testis barrier is not present at the level of the epididymis. The antibodies may neutralize the fertilizing ability of the spermatozoa as already shown with the hamster P26h and may confer an immunocontra-  
35   ceptive protection.



The peptide may be coupled to a carrier that may modulate the half-life of the circulating peptide. This may allow control on the period of contraceptive protection. The peptide-carrier may be emulsified in an adjuvant and administered by an appropriate immunization route.

In men under such an immunocontraceptive regimen, the circulating titer of anti-peptide antibodies may be an indication of contraceptive efficiency. Reversibility may be predicted by standard immunological determination of the titer of antibodies specific to the specific peptide.

- 24 - 24.06.00

WHAT IS CLAIMED IS:

1. Use of an antigenic fragment of a P34 protein for immunocontraception of a male or female subject, which comprises administering to said male or female subject an antigenic fragment of a P34 protein to elicit an immunocontraception response by said male or female subject.
2. The use according to claim 1, wherein said P34 protein is encoded by a sequence as set forth in SEQ ID NO:3, and wherein said antigenic fragment has an amino acid sequence selected from a group consisting of SEQ ID NO:4 and SEQ ID NO:5.
3. An immunocontraceptive vaccine for a male or female subject, which comprises an antigenic fragment of a P34 protein in association with a suitable pharmaceutically acceptable carrier, wherein said vaccine elicits an immunocontraception response by said male or female subject after its administration.
4. A probe as a marker for male or female fertility, which comprises a cDNA sequence capable of hybridizing under stringent conditions with a human acrosomal sperm protein P34.

$\frac{1}{12}$ 

	MK	LN	FS	XL	RA	LV	TG	AG	KG	IG	IG	XD	TA	KA	L
P26h															
Adipsin	--	--	--	G-	--	--	--	--	--	--	--	R-	-V	--	--

	XA	TE	KA	LG	XI	GP	VX	XL	VN	NA	AL	XX	XQ
P26h													
Adinsin	D-	--	--	--	G-	--	-D	L-	--	--	--	VM	I-

P26h	LY	PY	KX	RV	N
Adipsin	-G	-H	-I	--	--

# LEI

$2/12$ 

	1	2	3	4	5	6	7	8	9	10	11	12
--	---	---	---	---	---	---	---	---	---	----	----	----

1081

# Cyclophilin

fish

GTCCCTGGAGGTTGGCTGTAGGATTCAAGTGGCTTGCTCAGGCTGGG	47
ATCAAGGACACAGTGAGCAGATCAACCTTAACCTCAGCCCCCTCCC	92
CTCGCCACAGGAGGACACTGGTGTGTCAGCAGC ATG AAG CTG AAT	135
M K L N	4

TTTC ACT GGT CTC AGG GCT CTG GTG ACC GGG GCA GGG AGA GGG 177  
F T G L R A L V T G A G R G 18

ATT GGG CGA GGC ACT GCG AAA GCC CTG CAT GCC TCA GGA GCC 219  
I G R G T A K A L H A S G A 32

AAA GTG GTG GCC GTG TCA CTC ATC AAC GAA GAC CTG GTC AGC 261  
K V V A V S L I N E D L V S 46

CTG GCC AAA GAG TGT CCG GGC ATA GAG CCT GTG TGT GTG GAC 303  
L A K E C P G I E P V C V D 60

CTG GGT GAC TGG GAG GCC ACA GAG AAG GCA CTG GGC CGT ATT 345  
L G D W E A T E K A L G R I 74

GGC CCC GTG GAC CTG CTG AAC AAT GCG GCG GTG GCG CTA 387  
G P V D L L V N N A A V A L 88

# 713A

GTG CAG CCT TTC ATA CAG TCT ACC AAG GAG GTC TTT GAC AGG	429
V Q P F I Q S T K E V F D R	102

TCC	TTC	AAT	GTG	AAT	GTG	CGC	TCT	GTG	CTG	CAA	GTG	TCC	CAG	471
S	F	N	V	N	V	R	S	V	L	Q	V	S	Q	116

ATG	GTA	GCC	AAG	GGC	ATG	ATT	AAC	CGT	GGA	GTG	GCA	GGA	TCC	513
M	V	A	K	G	M	I	N	R	G	V	A	G	S	130

ATT GTC AAC ATC TCC AGC ATG GTG GCC TAT GTC ACC TTC CCT  
I V N I S S M V A Y V T F P

GGT CTG GCC ACG TAC AGC TCC ACC AAG GGT GCT ATA ACC ATG 597  
G L A T Y S S T K G A I T M 158

CTG ACC AAA GCC ATG GCC ATG GAG CTG GGA CCA TAC AAG ATC	639
L T K A M A M E L G P Y K I	172

CGG GTG AAC TCT GTA AAC CCT ACC GTG GTG CTG ACT GAC ATG	681
R V N S V N P T V V L T D M	186

GGC AAG AAA GTC TCT GCA GAC CCG GAA TTT GCC AAG AAG CTC	723
G K K V S A D P E F A K K L	200

Handwritten: 11-11-11



$\frac{6}{12}$ 

File 4A



7/12

P26h	ST	KG	AI	TMLT	KA	MAMELG	PY	KI	RV	NS	VN	PT	VV	LT	DMGK	KV	SA	DP	EF	AK	KL	200	
Adipsin	--	--	-M	--	--	--	--	-H	--	--	--	--	--	--	--	--	--	--	--	--	-R	--	200
C.Reductase	--	--	-M	--	-S	--	--	-H	--	--	--	--	--	--	A-	-R	S-	TS	--	-L	-R	--	200
P26h	KE	RH	PL	RK	FA	EV	ED	VV	NS	IL	FL	LS	DS	SA	ST	SG	SG	IL	VD	AG	YL	AS	244
Adipsin	--	--	--	--	--	--	--	--	--	--	--	--	-R	--	--	G-	--	--	--	--	--	--	244
C.Reductase	--	--	-M	--	--	--	--	--	--	--	--	--	-R	--	--	S-	F-	--	--	--	--	--	244

FIG. 4B

8/12

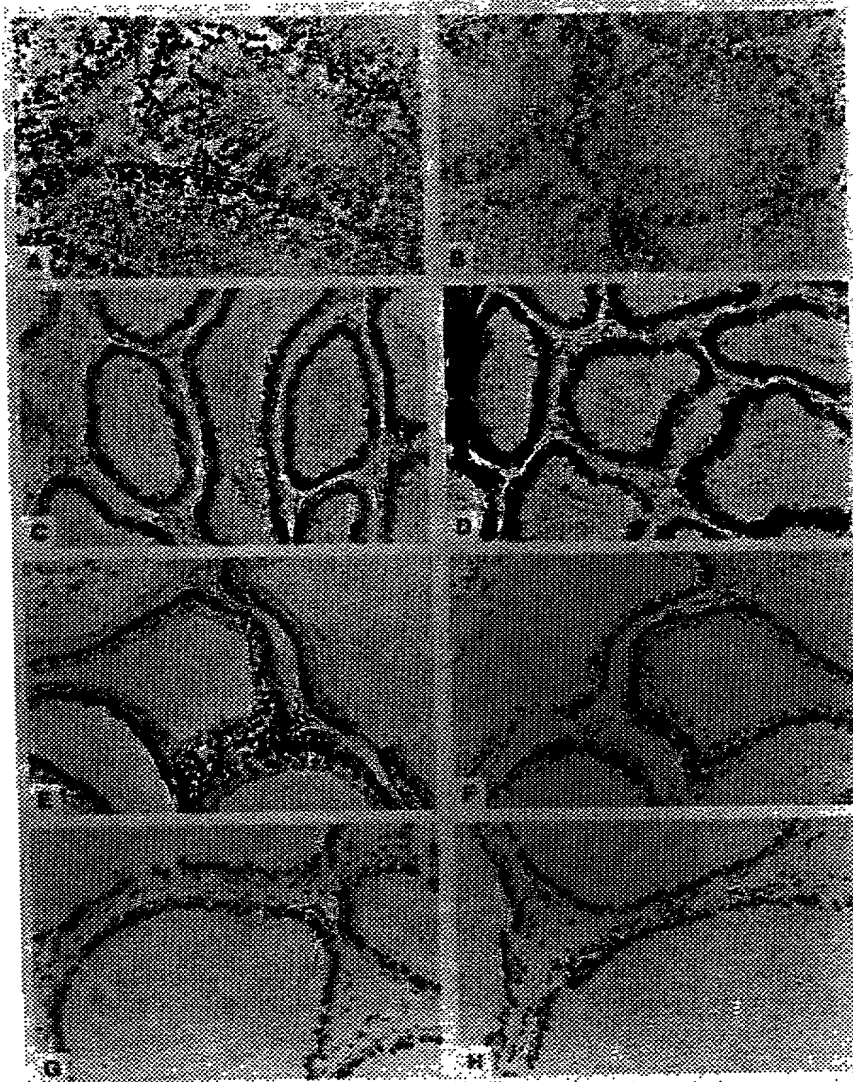


FIG. 5

9/12

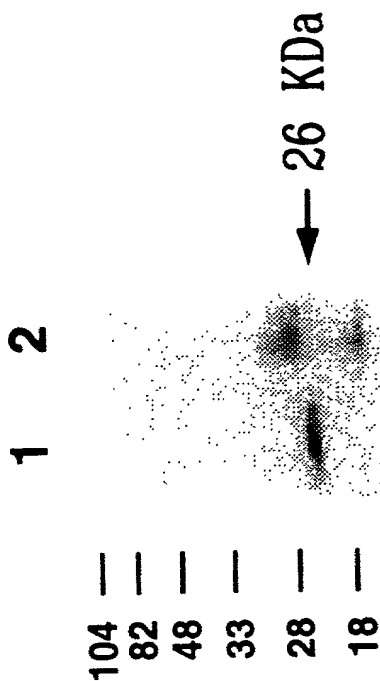


Fig. 6B

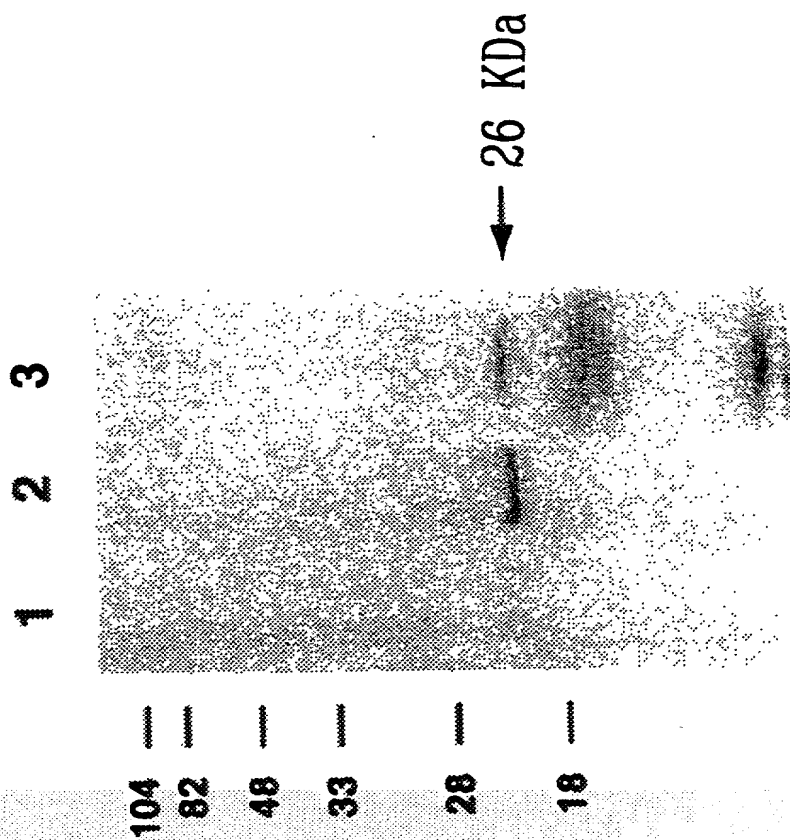
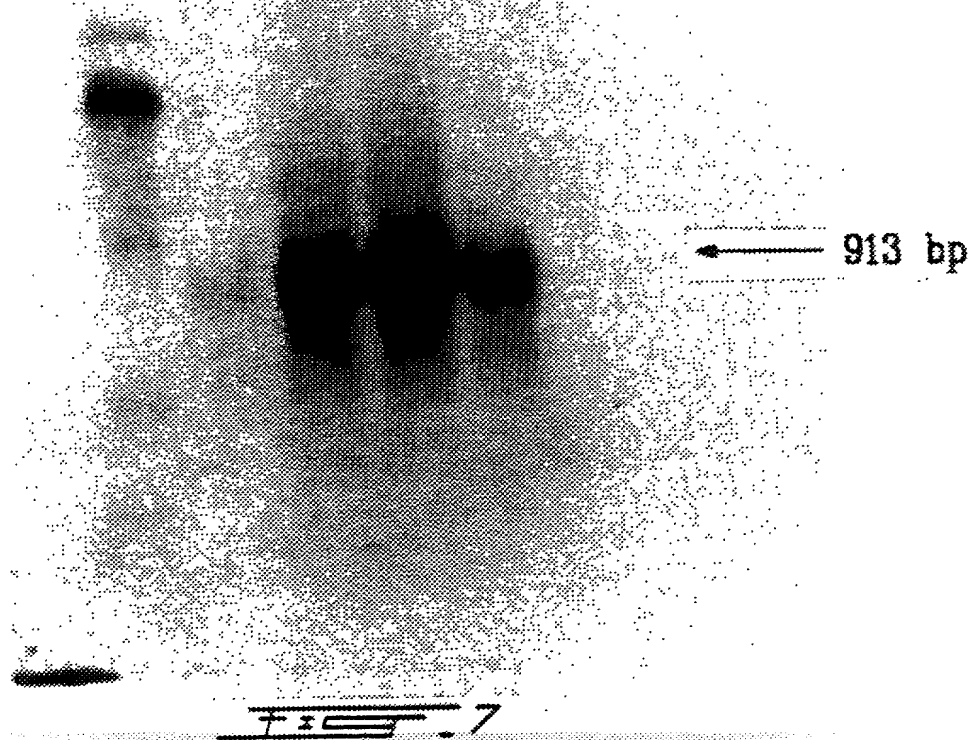


Fig. 6A

10/12

Epididymis

M	Testis	Caput	Corpus	Cauda
---	--------	-------	--------	-------



11/12

10 20 30 40 50 60 70 80  
 MKLNFTGLRALVTGAGRGIGRTAKALHASGAKVAVSLINEDLVSLAKECPGIEPVCVDLGDWEATEKALGRIGPVDLL  
 . . . . .  
 MELFLAGRRVLVTGAGKGIGRGTVQALHATGARVVAVSRTQADLDSLRECPGIEPVCVDLGDWEATERALGSGVPVDLL  
 10 20 30 40 50 60 70 80

90 100 110 120 130 140 150 160  
 VNNAVALVQPFIQSTKEVDFDRSFNVNVRSLQVSQMVAKGMINRGVAGSIVNISSMVAVVTFPGLATYSSTKGAITMLT  
 . . . . .  
 VNNAVALVQPFLEVTKEAFDRSFENVNLRVAVIQVSQIVARGVPGVAVNVSSQCSQRAVTHSVYCSSTKGALDMLT  
 90 100 110 120 130 140 150 160

FIG. 1 - BA

[illegible]

170		180		190		200		210		220		230		240				
KAMAMELG	PYKIR	VNSV	NPTV	VL	TDMG	KV	SADP	EFAK	KLKER	HPLR	KFAE	VEDV	VNSIL	FL	LDSSASTSGS	GILV	DAG	
..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	
KVMALE	LGPHKIR	VNAV	NPTV	VT	SMGQ	ATWSD	PHKAK	TMLN	RI	PLGK	FAE	VEH	VVNA	IL	FL	SDRSGMT	TGSTLP	VERG
170		180		190		200		210		220		230		240				

$$12/12$$

**SUBSTITUTE SHEET (RULE 26)**

YLAS

FWAC.

明日

<b>COMBINED DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63) AND POWER OF ATTORNEY</b>		Attorney Docket Number	13045-2US-1 FC
		First Named Inventor	Robert Sullivan et al.
		<i>Complete if known</i>	
		Application Number	
<input checked="" type="checkbox"/> Declaration Submitted with Initial Filing      OR <input type="checkbox"/> Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16(e)) required)		Filing Date	
		Group Art Unit	
		Examiner Name	

**As a below named inventor, I hereby declare that:**

My residence, post office address and citizenship are as stated below next to my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**ACROSOMAL SPERM PROTEIN AND USES THEREOF**

the specification of which

☐ is attached hereto.

OR

☒ was filed on 05/13/1999  
(mm/dd/yyyy)

as United States Application Number or PCT International Application Number PCT/CA99/00437

and was amended on 06/24/2000 (if applicable).  
(mm/dd/yyyy)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not claimed	Certified Copy Attached?	
				YES	NO
09/090,567	UNITED STATES	11/07/1997	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)

☐ Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

NO. 6400 P. 3/4

**COMBINED DECLARATION FOR UTILITY OR DESIGN  
PATENT APPLICATION (37 CFR 1.63) AND POWER OF ATTORNEY**

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT International application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)
PCT/CA99/00437	05/13/1999	

☐ Additional U.S. or PCT International application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto

**As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent Trademark Office connected therewith:**

☒ Customer Number: 020988



**020988**

**Direct all correspondence to:**



**020988**

## WHAT TO THINK OF

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

**Name of sole or First Inventor**

☐ A petition has been filed for this unsigned inventor

**Given Name (first and middle [if any])**

**Family Name or Surname**

**Robert**

**SULLIVAN**

**inventor's Signature**

Date \_\_\_\_\_

Residence: City

**Sto-Foy**

## Style

## Quibao

Country

**CANADA CAX**

## Citizenship

## Canadian

**Post Office Address**

**3201 Chemin St-Louis**

City

**Ste-Foy**

Province or State

**Québec**

Postal Code  
Or Zip

**G1W 1S1**

Country

**CANADA**

☒ Additional inventors are being named on the supplemental Additional Inventor(s) PTO/SB/02A attached hereto.



COMBINED DECLARATION FOR UTILITY OR DESIGN  
PATENT APPLICATION (37 CFR 1.63) AND POWER OF ATTORNEY

PTO/SB/02A (3-97)

**DECLARATION**

**ADDITIONAL INVENTOR(S)**  
Supplemental Sheet  
Page 1 of 1

**Name of Additional Joint Inventor, if any:**

☐ A petition has been filed for this unsigned inventor

Given Name (first and middle (if any))

Family Name or Surname

Bruno

DÉRUBÉ

Inventor's Signature

Date

Residence:

City

Loretteville

State

Québec

Country

CANADA CAX

Citizenship

Canadian

Post Office Address

75 du Buisson

City

Loretteville

Province  
or State

Québec

Postal Code  
Or Zip

G2A 1M7

Country

CANADA

**Name of Additional Joint Inventor, if any:**

☐ A petition has been filed for this unsigned inventor

Given Name (first and middle (if any))

Family Name or Surname

Christine

LÉGARE

Inventor's Signature

Date

Residence:

City

St-Rédempteur

State

Québec

Country

CANADA

Citizenship

Canadian

Post Office Address

294 Paquet

City

St-Rédempteur

Province  
or State

Québec

Postal Code  
Or Zip

G8K 1M3

Country

CANADA

**Name of Additional Joint Inventor, if any:**

☐ A petition has been filed for this unsigned inventor

Given Name (first and middle (if any))

Family Name or Surname

Christian

GAUDREAU

Inventor's Signature

Date

Residence:

City

St-Foy

State

Québec

Country

CANADA CAX

Citizenship

Canadian

Post Office Address

710 Horizon, Apt. 7

City

St-Foy

Province  
or State

Québec

Postal Code  
Or Zip

G1V 2X7

Country

CANADA

☐ Additional inventors are being named on the

supplemental Additional Inventor(s) PTO/SB/02A attached hereto.

## SEQUENCE LISTING

<110> IMMUCON INC.  
SULLIVAN, Robert  
BÉRUBÉ, Bruno  
LÉGARÉ, Christine  
GAUDREAU, Christian

<120> ACROSOMAL SPERM PROTEIN AND USES THEREOF

<130> 13045-2PCT FC/ld

<150> US09/090,567

<151> 1998-06-08

<160> 7

<170> FastSEQ for Windows Version 3.0

<210> 1

<211> 1081

<212> DNA

<213> Artificial Sequence

<220>

<221> CDS

<222> (124)...(856)

<223> p26h cDNA

<400> 1

```

gtccctggag gttggtgta ggattcaggt ggcttgetca ggctgggata aaggacacag      60
tgacgagatc aaccttaacc tcagccctc cctcgccac aggaggacac tgggtgtagc      120
agc atg aag ctg aat ttc act ggt ctc agg gct ctg gtg acc ggg gca      168
Met Lys Leu Asn Phe Thr Gly Leu Arg Ala Leu Val Thr Gly Ala
      1              5              10              15

ggg aga ggg att ggg cga ggc act gcg aaa gcc ctg cat gcc tca gga      216
Gly Arg Gly Ile Gly Arg Gly Thr Ala Lys Ala Leu His Ala Ser Gly
              20              25              30

gcc aaa gtg gtg gcc gtg tcactc atc aac gaa gac ctg gtc agc ctg      264
Ala Lys Val Val Ala Val Ser Leu Ile Asn Glu Asp Leu Val Ser Leu
              35              40              45

gcc aaa gag tgt ccg ggc ata gag cct gtg tgt gtg gac ctg ggt gac      312
Ala Lys Glu Cys Pro Gly Ile Glu Pro Val Cys Val Asp Leu Gly Asp
              50              55              60

tgg gag gcc aca gag aag gca ctg ggc cgt att ggc ccc gtg gac ctg      360
Trp Glu Ala Thr Glu Lys Ala Leu Gly Arg Ile Gly Pro Val Asp Leu
              65              70              75

ctg gtg aac aat gcg gcg gtg gcg cta gtg cag cct ttc ata cag tct      408
Leu Val Asn Asn Ala Ala Val Ala Leu Val Gln Pro Phe Ile Gln Ser
      80              85              90              95

```

acc aag gag gtc ttt gac agg tcc ttc aat gtg aat gtg cgc tct gtg 456  
 Thr Lys Glu Val Phe Asp Arg Ser Phe Asn Val Asn Val Arg Ser Val  
 100 105 110

ctg caa gtg tcc cag atg gta gcc aag ggc atg att aac cgt gga gtg 504  
 Leu Gln Val Ser Gln Met Val Ala Lys Gly Met Ile Asn Arg Gly Val  
 115 120 125

gca gga tcc att gtc aac atc tcc agc atg gtg gcc tat gtc acc ttc 552  
 Ala Gly Ser Ile Val Asn Ile Ser Ser Met Val Ala Tyr Val Thr Phe  
 130 135 140

cct ggt ctg gcc acg tac agc tcc acc aag ggt gct ata acc atg ctg 600  
 Pro Gly Leu Ala Thr Tyr Ser Ser Thr Lys Gly Ala Ile Thr Met Leu  
 145 150 155

acc aaa gcc atg gcc atg gag ctg gga cca tac aag atc cgg gtg aac 648  
 Thr Lys Ala Met Ala Met Glu Leu Gly Pro Tyr Lys Ile Arg Val Asn  
 160 165 170 175

tct gta aac cct acc gtg gtg ctg act gac atg ggc aag aaa gtc tct 696  
 Ser Val Asn Pro Thr Val Val Leu Thr Asp Met Gly Lys Lys Val Ser  
 180 185 190

gca gac ccg gaa ttt gcc aag aag ctc aag gag cgc cac cca ctg agg 744  
 Ala Asp Pro Glu Phe Ala Lys Lys Leu Lys Glu Arg His Pro Leu Arg  
 195 200 205

aag ttc gca gag gtg gag gac gtg gtc aac agc atc ctc ttc ctg ctc 792  
 Lys Phe Ala Glu Val Glu Asp Val Val Asn Ser Ile Leu Phe Leu Leu  
 210 215 220

agc gac agc agc gcc tct acc agc ggc tct ggc atc ctg gtg gac gct 840  
 Ser Asp Ser Ser Ala Ser Thr Ser Gly Ser Gly Ile Leu Val Asp Ala  
 225 230 235

ggt tac ctg gcc tcc t agacggccca ggtgcagggg actcctggag acttccctgg 896  
 Gly Tyr Leu Ala Ser  
 240

cctcaccctt acatcaagac cccgccttca acccaaccca ataattttgt tcgaatcctg 956  
 tagagcccca cccacacac atccatcccc aacttttagac tccgggatcc cgccattcca 1016  
 taccagctat gctgagataa tttgattaaa taagtatccc aaaccacaaa aaaaaaaaaa 1076  
 aaaaa 1081

&lt;210&gt; 2

&lt;211&gt; 244

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;400&gt; 2

Met Lys Leu Asn Phe Thr Gly Leu Arg Ala Leu Val Thr Gly Ala Gly  
 1 5 10 15  
 Arg Gly Ile Gly Arg Gly Thr Ala Lys Ala Leu His Ala Ser Gly Ala  
 20 25 30  
 Lys Val Val Ala Val Ser Leu Ile Asn Glu Asp Leu Val Ser Leu Ala  
 35 40 45

Lys Glu Cys Pro Gly Ile Glu Pro Val Cys Val Asp Leu Gly Asp Trp  
 50 55 60  
 Glu Ala Thr Glu Lys Ala Leu Gly Arg Ile Gly Pro Val Asp Leu Leu  
 65 70 75 80  
 Val Asn Asn Ala Ala Val Ala Leu Val Gln Pro Phe Ile Gln Ser Thr  
 85 90 95  
 Lys Glu Val Phe Asp Arg Ser Phe Asn Val Asn Val Arg Ser Val Leu  
 100 105 110  
 Gln Val Ser Gln Met Val Ala Lys Gly Met Ile Asn Arg Gly Val Ala  
 115 120 125  
 Gly Ser Ile Val Asn Ile Ser Ser Met Val Ala Tyr Val Thr Phe Pro  
 130 135 140  
 Gly Leu Ala Thr Tyr Ser Ser Thr Lys Gly Ala Ile Thr Met Leu Thr  
 145 150 155 160  
 Lys Ala Met Ala Met Glu Leu Gly Pro Tyr Lys Ile Arg Val Asn Ser  
 165 170 175  
 Val Asn Pro Thr Val Val Leu Thr Asp Met Gly Lys Lys Val Ser Ala  
 180 185 190  
 Asp Pro Glu Phe Ala Lys Lys Leu Lys Glu Arg His Pro Leu Arg Lys  
 195 200 205  
 Phe Ala Glu Val Glu Asp Val Val Asn Ser Ile Leu Phe Leu Leu Ser  
 210 215 220  
 Asp Ser Ser Ala Ser Thr Ser Gly Ser Gly Ile Leu Val Asp Ala Gly  
 225 230 235 240  
 Tyr Leu Ala Ser

<210> 3  
 <211> 912  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> P34 cDNA

<400> 3  
 gacaaaagct ggagctccac cgcgggtggcg gccgctctag aactagtgga tcccccgggc 60  
 tgcaggaatt cggcacgagc cgacatggag ctgttcctcg cgggcccgcg ggtgctggtc 120  
 accggggcag gcaaaggat agggcgcggc acggtccagg cgctgcacgc gacggggcgcg 180  
 cgggtggtgg ctgtgagcgc gactcaggcg gatcttgaca gccttgtccg cgagtgcccg 240  
 gggatagaac cgtgtgtcgt ggacctgggt gactgggagg ccaccgagcg ggcgctgggc 300  
 agcgtgggccc ccgtggacct gctggtgaac aacgcccgtg tcgccctgct gcagcccttc 360  
 ctggaggtca ccaaggaggc ctttgacaga tcctttgagg tgaacctgcg tgcgggtcatc 420  
 caggtgtcgc agattgtggc caggggctta atagcccggg gaggtaaccagg ggccatcgtg 480  
 aatgtctcca gccagtgtc ccagcgggca gtaactaacc atagcgtcta ctgctccacc 540  
 aagggtgccc tggacatgct gaccaagggt atggccctag agctcggggc ccacaagatc 600  
 cgagtgaatg cagtaaacc cactgtggtg atgacgtcca tgggccaggc cacctggagt 660  
 gacccccaca aggccaagac tatgtgaac cgaatcccac ttggcaagtt tgctgaggtg 720  
 gagcacgtgg tgaacgccat cctctttctg ctgagtgacc gaagtggcat gaccacgggt 780  
 tccactttgc cggtggaaag gggcttctg gctgctgag ctccctccac acacctcaag 840  
 ccccatgccg tgctcatcct acccccattc cctccaataa acctgattct gctcccaaaa 900  
 aaaaaaaaaa aa 912

<210> 4  
 <211> 11  
 <212> PRT  
 <213> Artificial Sequence

&lt;220&gt;

&lt;223&gt; P34 antigenic fragment

&lt;400&gt; 4

Met Glu Leu Phe Leu Ala Gly Arg Arg Val Leu  
1 5 10

&lt;210&gt; 5

&lt;211&gt; 11

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;400&gt; 5

Cys His Lys Ala Lys Thr Met Leu Asn Arg Ile  
1 5 10

&lt;210&gt; 6

&lt;211&gt; 21

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; cDNA for use as primer

&lt;400&gt; 6

gtgacagggg cagggaaagg g

21

&lt;210&gt; 7

&lt;211&gt; 21

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; cDNA for use as primer

&lt;400&gt; 7

gcaactgagc agactaggag g

21